DNA aptamer-based detection of prostate cancer

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The use of aptamers in biosensing have gained considerable attention as an attractive alternative to antibodies because of their unique properties such as long term stability, cost effectiveness and tunability to various applications. Among various cancers, early diagnosis of prostate cancer (PCa) is one of the biggest concerns for ageing men worldwide. One of the most commonly used biomarker for PCa is prostate specific antigen (PSA), which can be found in elevated levels in patients with cancer. In this review, a presentation on the gradual transition of research from antibody-based to aptamer-based biosensors is presented specifically for PSA. A brief description on aptamer-based biosensing for other PCa biomarkers is also presented. Special attention is given to electrochemical methods as analytical techniques for development of simple, sensitive and cost effective biosensors. The review also focuses on different surface chemistries exploited for fabrication and their application with clinical samples. Utilization of aptamers provides a promising tool for development of point-of-care biosensors for early detection of prostate cancer. In the view of the unmatched upper hand of aptamers, future perspectives are also discussed, not only in the point of care format but also in other novel applications.

Keywords: DNA aptamer, biosensor, electrochemical detection, prostate specific antigen, prostate cancer, surface chemistry

Introduction

Prostate cancer (PCa) is a type of cancer that develops in the prostate gland, which is a part of a male reproductive system. PCa is the most commonly diagnosed cancer amongst men in Europe and the United States and is the second worldwide leading cause of morbidity. It has been reported that PCa is predominant in older men above the age of 50 (Kirk, 1997; Hoffman, 2011) and among black men (Stanford et al., 1999; Greenlee et al., 2000). It has been also projected that PCa will be the most common cancer by 2030 in the UK (Greenlee et al., 2000; Jeong et al., 2010).

Most of the PCa generate in the epithelium cells (Bostwick, 1989). As androgens regulate cell division of the gland epithelium (Ross et al., 1998), these hormones are believed to be the main cause of PCa. However, a study demonstrating a consistent correlation between androgens and prostatic carcinogenesis has not yet been reported to date and the precise causes that lead to PCa are still not well understood (Kufe et al., 2003).

PCa often develops very slowly and the lack of symptoms during the early stages of the disease leads to a late diagnosis of the tumour. Moreover, if diagnosed at a late stage, no effective treatments are currently available for its cure. In many cases PCa does not show any clinical manifestation during the lifetime of a patient, who might die for non-related PCa causes. However, for those patients that develop a more aggressive cancer form, PCa cells can break away from a prostate tumour and metastasise. Since the prostate is well connected to numerous lymph nodes, the spread is easy and some of the most common sites of PCa metastatic process are bones (Chou & Simons, 1997).

Current detection methods

There is no solitary test for the diagnosis of PCa. Moreover, all the tests which are used to diagnose have pros and cons which are usually discussed by the doctors with their patients. The most commonly used methods for PCa detection are: digital rectal examination (DRE), transrectal ultrasound (TRUS), biopsy and PSA blood test.

In DRE, a doctor inserts a gloved finger into the rectum and examines for bumps or swelling of the prostate gland. It is an inexpensive method and can also detect PCa

irrespective of changes in the level of prostate specific antigen (PSA) in blood. Accuracy of diagnosis can be increased when DRE is combined with PSA tests and biopsy results (Uzzo et al., 1995; Basler & Thompson, 1998; Jeong et al., 2010). In comparison to DRE, in the TRUS method an ultrasound probe is inserted into the rectum, emitting energy sound waves to image the prostate gland. It is a very useful tool to understand pathology of tumours and in guiding needle biopsies for sampling of tissue (Aus et al., 1996; Irani et al., 1997). For a biopsy, a small section of the tissue is removed through the rectum using a needle and is microscopically examined by pathologists. It requires a high number of samples from the prostate making it a painful protocol. Not only the results from biopsies are controversial, there is also a high risk of severe infections with subsequent biopsies (Jeong et al., 2010; Loeb et al., 2013).

The most frequently used test for PCa screening is the quantification of levels of PSA in blood. If PSA levels are higher than the cut off levels of 4 ng/ml, biopsy procedures are considered (Catalona et al., 1991, Jeong et al., 2010; Savory et al., 2010). However, the levels of PSA in blood in ageing men can also be raised due to other factors like benign prostatic hyperplasia (BPH) and prostatis, which could lead to an over-diagnosis in men (Carter et al., 1992). Consequently, due to faulty diagnosis, patients undergo biopsy surgery making PSA testing a controversial diagnostic tool. Due to these controversies with PSA testing, in May 2012 the US Preventative Services Task Force recommended against PSA screening in all men. This emphasized the need for more reliable biomarkers for diagnosis of the disease (Moyer, 2012).

Prostate specific antigen (PSA): a PCa biomarker

PSA belongs to the family of kallikrein proteins which are defined as serine proteases. There are about 15 kallikrein family members that have been identified in humans. PSA is the only kallikrein specific to prostate (hK3). Pancreatic renal kallikrein (hK1) and human glandular kallikrein (hK2), which are androgen regulated, are also expressed in the prostate (Balk et al., 2003).

PSA is synthesised in its inactive form: a 244 amino acid long protein called pro-PSA. Pro-PSA is cleaved from the N terminus in the prostate by the hK2 enzyme leading to active PSA which is a 237 amino acid long protein (Takayama et al., 1997). The active PSA is a 30 kDa protein which can be found in both serum and semen of men. PSA is present in semen

in the range of 0.5 - 2 mg/ml and its physiological role is to de-coagulate semen by breaking down the proteins semenogelin I and II (Lilja et al., 1987; Lövgren et al., 1999). In prostate cancer there is release of both active PSA and pro-PSA due to rupture of the basal membrane. Moreover, internally cleaved forms of PSA (with no enzymatic activity) also enter the blood stream but remain un-complexed and are taken into the free PSA (fPSA) count. However, when active PSA enters the blood stream it becomes immediately complexed with protein inhibitors. Most of the assays employing antibodies measure the total amount of PSA (tPSA) (Takayama et al., 1997).

Many studies reported that PSA levels are directly proportional to the stage of the cancer and to the volume of the tumour (Stamey et al., 1987; Grossklaus et al., 2002; Pinsky et al., 2007; Lilja et al., 2008). PSA detection results are nowadays highly sensitive (Madu & Lu, 2010) and reasonably inexpensive. Moreover PSA testing is a more accepted procedure by patients compared to DRE and this has augmented the early detection of PCa (Balducci et al., 1997). However, even though PSA testing induced a decrease in PCa mortality of 20% its screening led to over-diagnosis and over-treatment (Andriole et al., 2009) of patients that would have not been clinically affected by the tumour during their lifetime. Over diagnosis can, in fact, lead to unnecessary treatments and increase the state of anxiety in patients. Conversely, clinicians are not able nowadays to discriminate between a harmless or lethal form of prostate cancer and so to decide whether the patient needs a treatment. Once a prostate cancer has been definitively treated, PSA screening is the most reliable and fast means that enable to detect a contingent recurrence of the tumour (Lilja et al., 2008).

With the shortcomings of the current tests for PCa, including PSA testing, there is a concerted effort to look for alternatives. However, it would be a challenge to replace PSA entirely due to its minimally invasive nature and low cost. Instead, there is a pressing need to look for other biomarkers to complement PSA that can increase the specificity and sensitivity of PCa screening and inform prognosis and treatment courses.

One path currently being looked at when a high level of PSA is detected in patients with cancer, is to differentiate PSA into different forms namely free PSA (fPSA) and total PSA (tPSA) and quantify them independently. One of the approaches is to measure the ratio of free PSA to total PSA in the blood. It has been proven, in fact, that the levels of fPSA are lower in patients with PCa than in patients with BPH (Christensson et al., 1993), which can thus be an indication of the aggressiveness of the cancer. However, the method can cause false negative results as the amount of fPSA can be higher in patients with larger prostate volume (Stephan et al., 1997; Catalona et al., 1998). Nevertheless, the ratio of free to total

PSA when combined with the total PSA levels increases the confidence of the diagnosis (Velonas et al., 2013).

Pro-PSA

Several studies are also focused on the detection of a distinct form of free PSA, called proenzyme PSA (pro-PSA). Pro-PSA is an enzymatically inactive precursor of PSA obtained by co-translational removal of an amino-terminal leader. The N-terminal of pro-PSA can be cleaved at various positions resulting in different forms of pro-PSA. Pro-PSA truncated between the third and second amino acid is called [-2]pro-PSA and is believed to provide a better discrimination between cancerous and benign form of prostate disorders (Mikolajczyk et al., 2001; Mikolajczyk et al., 2004). Increased values of other forms of pro-PSA ([-5] and [-7]) have also been associated to PCa. A truncated precursor form of prostate-specific antigen is therefore a more specific serum marker of prostate cancer.

PSA density

A better discrimination of BPH from PCa might be achieved by measuring the ratio of PSA to prostate volume. However, this parameter called PSA density showed contradictory evidence on the tumour aggressiveness and malignity (Stamey et al., 1987; Ohori et al., 1995). Furthermore, in order to obtain prostate volume values, TRUS is required in addition to the standard PSA test with a consequent discomfort for patients as well as an increase in the cost and time required to perform the test. For these reasons PSA density has not been extensively employed as a routine test for PCa.

PSA velocity and PSA doubling time

PSA velocity refers to the rate of serum PSA increase over time while PSA double time refers to the time required for a given PSA level to be doubled. As the previous PSA derivatives, also PSA velocity can be used to distinguish a prostate cancer from a BPH (Carter et al., 1992). Both PSA velocity and PSA double time are used to monitor the recurrence of the tumour after treatment (D'Amico et al., 2004; D'Amico et al., 2005). Again, some studies compared the responses from PSA velocity and PSA double time with biopsy results demonstrating how these two PSA derivatives can fail the diagnosis (Melichar, 2012)

Age-specific PSA reference ranges

Since the level of PSA increases with the age of men, scientists studied this correlation in order to obtain a median value of PSA for given ranges of age. By comparing the PSA level with the median PSA for that patient's age (age-specific PSA) a better choice might been taken before ordering biopsies (Loeb & Catalona, 2007).

Oligonucleotide Aptamers

In recent years, a range of assays for PSA detection such as electrochemical assays (Okuno et al., 2007; Panini et al., 2008), enzyme linked immunosorbent assays (Acevedo et al., 2002), cantilever assays (Wee et al., 2005), and chemiluminescent immunoassays (Albrecht et al., 1994; Seto et al., 2001) have been developed. These assays are mostly based on antibodies as recognition elements. One of the alternatives to antibodies is aptamers which can offer several advantages with respect to the former. However, an enormous research is being carried out to prove if antibodies can be replaced by aptamers to develop a real biosensor for clinical applications. The scope of this review is to highlight the major developments on PSA aptasensors and their potential to be used with real clinical blood samples.

Oligonucleotide aptamers are single stranded DNA or RNA sequences that can bind to a target molecule with high specificity and affinity. Aptamers had already been widely used in drug delivery applications and are now being extensively studied as new emerging bioreceptors for biosensors (termed aptasensors) (Hianik & Wang, 2009; Iliuk et al., 2011). Aptamers have shown comparable or even stronger binding than antibodies towards a broad range of targets (e.g. proteins, peptides, amino acids, drugs, whole cells, etc.), especially with the development of novel selection technologies (Xiao et al., 2005). The high affinity of the aptamers towards the target molecule is defined by their capability of undergoing conformation changes upon the binding event (Hermann & Patel, 2000; Song et al., 2008; Hianik & Wang, 2009). Although using aptamers have many added advantages over antibodies, they still need careful consideration while fabricating a biosensor. For instance, binding of an aptamer to protein might be affected by changing buffer conditions. Also, as aptamers are oligonucleotide sequences, special care is needed as they are sensitive to DNase

and RNase activity. Furthermore, the k_d value of aptamers is often not as good as that for antibodies.

Aptamers are developed using an *in vitro* selection process based on Systematic Evolution of Ligands by EXponential enrichment (SELEX) (see fig. 1). Briefly, it consists of three steps that are repeated systematically in order to identify the oligonucleotide sequence that binds better to the target. The first step is called library generation, where a library consisting of random DNA or RNA sequences (usually 30-40 base-pairs long) flanked by the primer binding site are used. The library is then incubated with the target molecule. Thereafter, the target bound library is separated from unbound library. Finally, the target-bound library is amplified using polymerase chain reaction (PCR) to create a new library to be used in the next round. Aptamers binding and conformation characteristics are identified using various biological assays (Syed & Pervaiz, 2010; Liu et al., 2012).

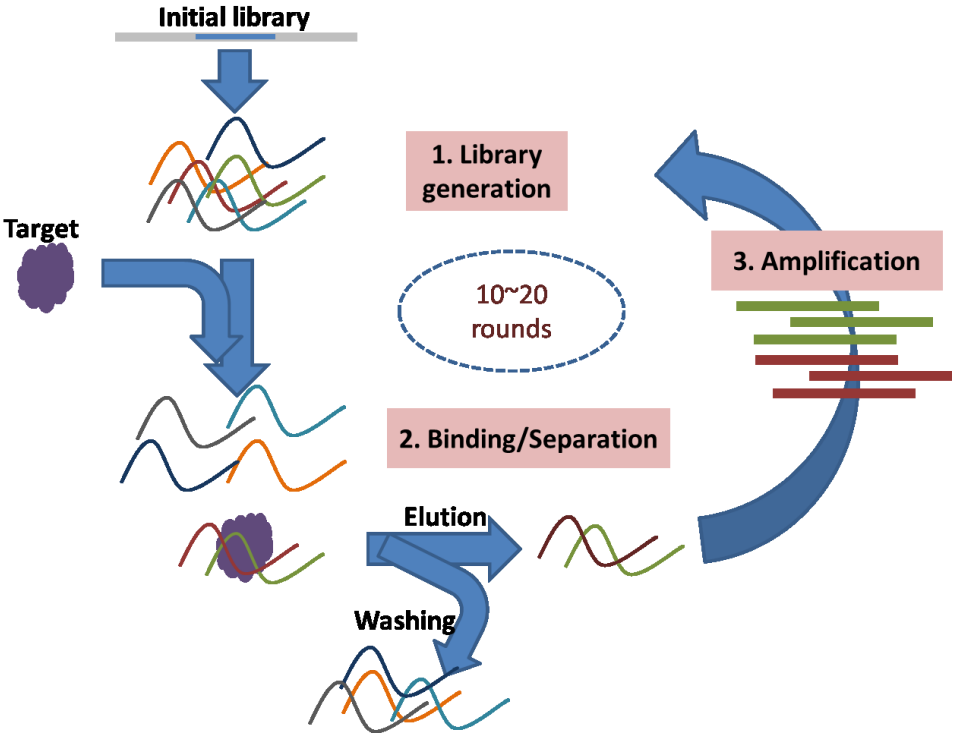

Fig. 1. The general SELEX protocol. Starting with a random library followed by incubation with the target. Later the bound sequences are separated and further amplified for the next round of selection. Adapted from Song et al. (2008).

There has been an intense interest in understanding the in-depth of ligand-binding and conformational properties of aptamers. Aptamers have many advantages over antibodies, making them very important molecular tools for both diagnostics and therapeutics. For instance, selection of aptamers is an *in vitro* process and they can be raised to a wide variety of targets ranging from small molecules and toxins to large proteins and even whole cells. Secondly, aptamers, once selected, can be synthesised with high purity and reproducibility. Also, as compared to antibodies, aptamers are usually highly chemically stable. Furthermore, they can undergo significant conformational changes in their structure upon binding with the target – a feature which can be exploited for biosensing applications. This offers great flexibility to design novel biosensors (Clark & Remcho, 2002; Tombelli et al., 2005; Willner & Zayats, 2007; Mairal et al., 2008; Song et al., 2008; Liu et al., 2012).

PSA detection

PSA is currently detected in dedicated laboratory settings using automated analysers running antibody-based assays which are generally expensive and time consuming (Lin & Ju, 2005; Healy et al., 2007). Cost effective, easy to use and possibly portable devices are required in order to allow more powerful tools for early detection of prostate cancer. To date, researchers have exploited several techniques for PSA detection such as optical (Besselink et al., 2004; Huang et al., 2005; Cao & Sim, 2007), piezoelectric (Weeks et al., 2003; Wee et al., 2005) and electrochemical (Sarkar et al., 2002; Fernández-Sánchez et al., 2004; Liu et al., 2013).

Although label-free-based biosensors can provide many advantages, label-based approaches are still intensively studied and can offer interesting features such as low limit of detection due to amplification strategies. An interesting magnetic bead-based detection system for PSA detection was developed by Zani et al. (2009): paramagnetic microparticles were adsorbed on an array of screen-printed electrodes and PSA was sandwiched in between two antibodies on the beads; the alkaline-phosphatase-labelled secondary antibody could be detected with differential pulse voltammetry (DPV) to achieve a detection limit of 1.4 ng/ml. A limit of detection as low as 0.5 pg/ml in undiluted serum samples was obtained by Mani et al. (2010) by combining a multienzyme-labelled immunoassay with gold nanoparticles sensing surface: in this case the secondary antibody was bound to micromagnetic HRP-labelled beads, which massively amplified the current signals for a very low PSA detection

limit. A similar detection technique was improved and integrated in a microfluidic system by Chikkaveeraiaha et al. (2011) reaching an even lower detection limit. A fascinating electrochemiluminescence-based immunoassay was developed by Sardesai et al. (2011) for both PSA and interlukin 6 (IL-6) by using single-wall carbon nanotubes (SWCNT) fabricated on microwells and a sandwich assay where the secondary PSA antibody was functionalized with RuBYP-Silica particles: the detection limit achieved was of 1 pg/ml for PSA.

Label-free electrochemical sensors for PSA detection

Electrochemical techniques are widely employed in biosensing devices as they can be highly sensitive, simple to perform and cost effective. An electrochemical biosensor involves an electrode surface that is functionalised with a molecular recognition element for sensing biomolecules. Binding of an analyte to this element results in an electrical change in current transfer (amperometric), voltage (potentiometric and field effect transistors), impedance (impedimetric), conductivity (conductometric) or ion charge across the electrode, which can be quantified and correlated to the amount of analyte captured.As mentioned in the previous sections, most biosensors for PSA detection currently available are antibody-based. Amongst the antibody-based electrochemical sensors, particularly important results are the ones using label-free systems. Arya & Bhansali (2012) developed a gold biosensor modified with a cysteamine self-assembled monolayer (SAM) for PSA detection. Li et al. (2005), on the hand, employed In_2O_3 nanowires and carbon nanotubes. Electrochemical impedance spectroscopy (EIS) based sensors have been reported by Chiriacò et al. (2013) and Chornokur et al. (2011). The former exploits a combined use of two different antibodies for both free and total PSA, while the latter reported on a miniaturized sensor obtained with photolithographic techniques using a single monoclonal antibody. Another label-free antibody-based sensor which uses a polycrystalline silicon field-effect transistor was reported by Huang et al. (2013).

Aptasensor for PSA detection

An aptasensor biosensor comprises an aptamer as a biorecognition element (Lim et al., 2009). Aptasensors can be integrated with different sensing techniques such as electrochemical, optical, and mass sensitive. Among these varied techniques, electrochemical aptasensors have been fabricated using several detection techniques, namely EIS, potentiometry and differential pulse voltammetry (DPV) (Cho et al., 2009; Clark & Remcho,

2002; Feng et al., 2008; Ikebukuro et al., 2005; Liu et al., 2012; Numnuam et al., 2008; Wang et al., 2007; Xu et al., 2005). For detection of PSA, both RNA and DNA aptamers have been developed, although there are only a handful of reports on PSA biosensors using aptamers. A summary of aptamer-based biosensors for PCa detection is presented in table 1.

Table 1. Performance comparison of different aptasensors for PCa detection

Method	*Material*	*Biomarker*	*Detection limit*	*Reference*
QCM-D/EIS	Gold	PSA	-	Formisano et al., 2014
EIS	Gold	PSA	1 ng/ml	Jolly et al., 2014
Optical	AuNPs	PSA	32 pg/ml	Chen et al., 2012
DPV/CV	AuNPs@GMCs	PSA	0.25 ng/ml	Liu et al., 2012
EIS	Gold	PSMA cells	-	Min et al., 2010

The first aptamer developed was a RNA aptamer (Jeong et al., 2010) that has been used to demonstrate the recognition of active PSA. Following that, a DNA aptamer was developed using a genetic algorithm with post-SELEX screening against PSA (Savory et al., 2010). To date, there is no reported literature on the application of RNA aptamers for PSA biosensing, which could be due to the long length of the sequence making it difficult to synthesise commercially.

DNA based PSA aptamer has been combined with different sensing techniques with sensitivities ranging from pg/ml to ng/ml. Chen et al. (2012) were the first to report the use of PSA aptamer to develop an optical based aptasensor. The conjugation of gold nanoparticles (AuNPs) with DNA aptamers were used to develop an aptasensor based on resonance light scattering (RLS) spectral assay. The novel technique relied on changes in resonance light scattering on binding of PSA to the aptamer, with a detection limit of 32 pg/ml. Thiolated DNA aptamers were immobilized on AuNPs and then a blocking step with BSA was performed prior the use of the complex AuNPs-aptamers with PSA samples. In this configuration, the gold surface of the nanoparticles was covered by the flexible aptamer structure and as a result no aggregation of particles occurred in absence of PSA. In the presence of PSA, aptamer-PSA complexes were formed and the aptamers undergo a conformational change in their structure from flexible to rigid. The changes in aptamer

conformation exposed some parts of the AuNPs that were thus available to form AuNPs aggregates upon addition of potassium chloride. This resulted in an increase in the RLS signal. The assay exhibited good sensitivity and selectivity towards PSA and tests made on human blood samples showed results comparable to those obtained with ELISA (relative deviation < 7%).

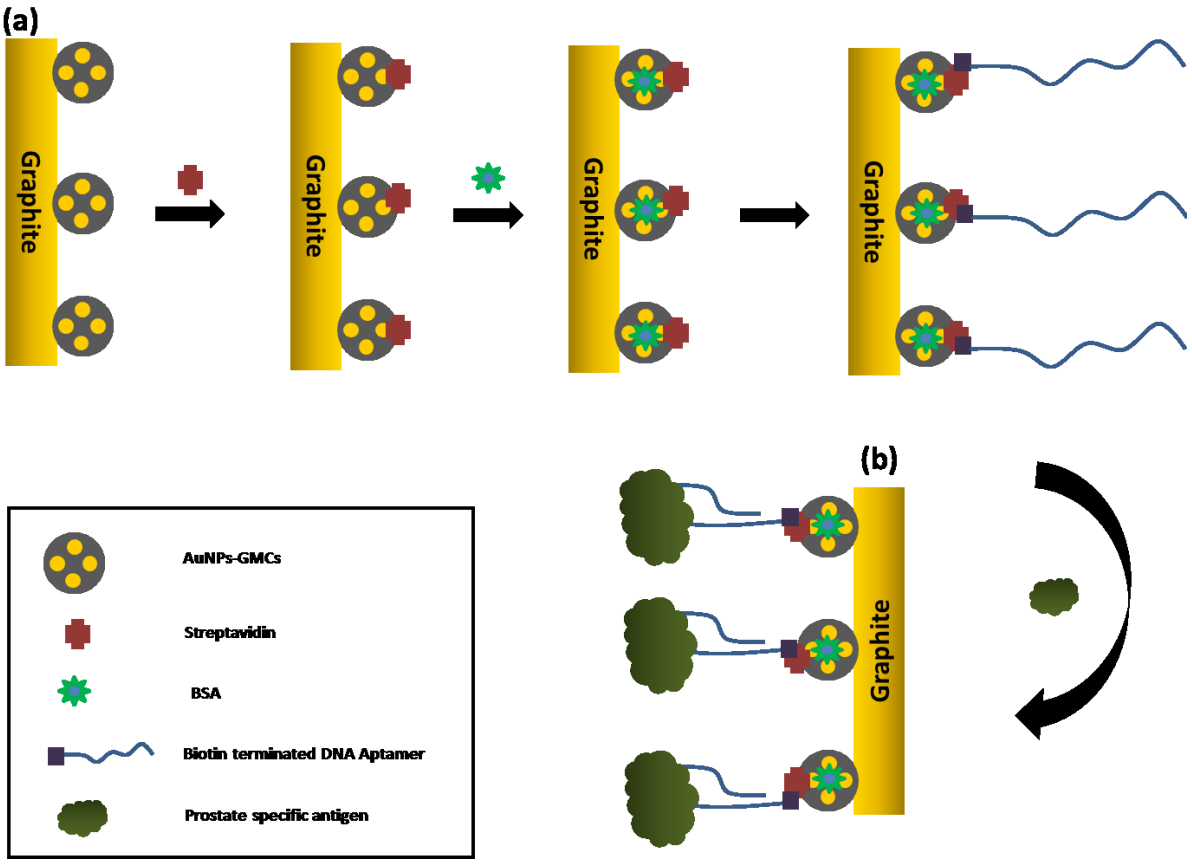

Fig. 2. Schematic illustration of fabrication process of the aptasensor based on gold nanoparticles encapsulated by graphitized mesoporous carbon (a); PSA detection (b). Adapted from Liu et al. (2012).

With regards to electrochemical aptasensor, modification of the electrode surface is one of the biggest fields of investigation. Research is typically focused on finding the most suitable recognition platform to give a stable organization to the sensor interface leading to optimized binding efficiency and signal outcome (Lee et al., 2005; Putzbach & Ronkainen, 2013). Liu et al. (2012) applied aptasensors based on amplification via AuNPs and

graphitized mesoporous carbon (GMCs) combined with streptavidin-biotin system for electrochemical detection of PSA (see fig. 2). GMCs encapsulated AuNPs formed the first layer on cleaned pyrolytic graphite electrode followed by coating with streptavidin. All the non-specific sites were blocked with bovine serum albumin (BSA). Finally, biotinylated DNA aptamers were allowed to react with streptavidin immobilized on electrode surface. The fabricated aptasensor was then used to capture PSA which was measured via differential pulse voltammetry (DPV). The limit of detection of the aptasensor was 0.25 ng/ml with high specificity to PSA. In spite of high sensitivity and specificity, the fabrication procedure which is a layer-by-layer development of sensor surface is quite complex, which may be a drawback in fabricating a cost effective sensor. The group also used Electrochemical Impedance Spectroscopy (EIS) to characterize the layer-by-layer fabrication of the aptasensor.

Electrochemical Impedance Spectroscopy is one of the most promising electrochemical techniques for DNA-based approaches but requires a careful design in order to optimize its signal. Particularly important for EIS biosensors is the formation of a well-organized self-assembled monolayer (SAM) which allows an optimal charge transfer to occur. For successful EIS measurements, it is necessary to have a good and reliable SAM layer on the gold electrode surface. One of the most accepted approaches to achieve this goal is by alkanethiol chemistry. Alkanethiols can be easily adsorbed and form SAMs (Love et al., 2005) on a clean gold surface through thiol bonds (see fig. 3). It has been reported that longer alkane chains give a more compact structure with minimal defects (Campuzano et al., 2006). Among different configuration of SAM, a mixed SAM of 11-Mercaptoundecanoid acid (MUA), $HS(CH_2)_{10}COOH$, and 6-Mercapto-1-hexanol (MCH), $HS(CH_2)_6OH$, exhibited reasonable starting impedance values and improved reliability (Herne & Tarlov, 1997). In order to gather or to enhance the extent of a measureable signal of the recognition event occurring on the working electrode, marker molecules such as redox couples, are exploited. The recognition events that happen on the SAM not only modify the charge transfer processes between redox couples present in the measurement solution and the sensor surface but also affect the double layer at the sensor interface. Both these events cause a change in the system charge transfer resistance (R_{ct}) which can then be measured by using an appropriate equivalent circuit.

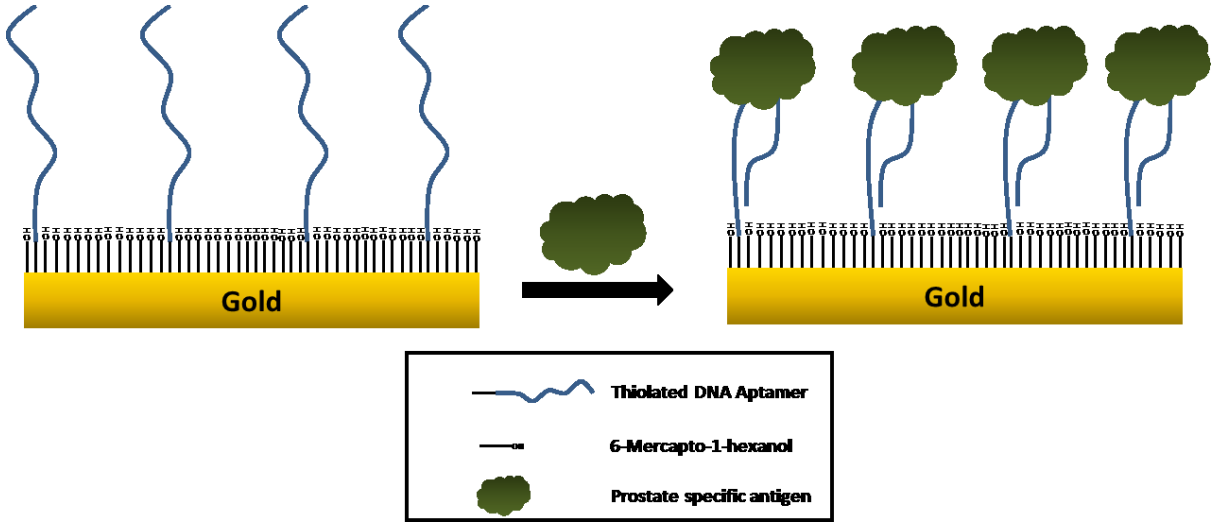

Fig. 3. Schematic illustration of fabrication process of the aptasensor with 6-mercaptohexanol and thiolated DNA aptamer.

In EIS measurements using PSA aptamers, Jolly et al. (2014) and Formisano et al. (2014) reported a reduction in charge transfer resistance (R_{ct}) upon binding of PSA to the immobilised DNA aptamers. This decrease is contradictory to what has been reported in the literature for PSA where an increase of R_{ct} has been observed (Liu et al., 2012), even though these studies used EIS mainly to characterize the bio-recognition layer and not for dose response determination. A reduction of R_{ct} upon aptamer-analyte interaction has also been reported for a different aptasensor using a lysozyme aptamer, where the reduction in charge transfer resistance upon binding of lysozyme to its specific DNA aptamer was attributed mainly due to screening of charges on DNA (Rodriguez et al., 2005). The reduction of R_{ct} could arise from two reasons: firstly, upon binding, PSA might screen the charges of the DNA aptamer; secondly, as PSA is also a charged protein, it could be that more positive charges are exposed because of the protein architecture itself. Consequently, as there is screening of charges of DNA, there is a reduction on electrostatic barrier to the ferro/ferricyanide anions towards the electrode surface, leading to lowering of the R_{ct} value of the system.

Earlier reports on DNA detection using DNA (Keighley et al., 2008a) and PNA probes (Keighley et al., 2008b) have demonstrated the importance of optimization of the oligonucleotide probe surface coverage in order to have efficient binding. On the same grounds, Formisano et al. (2014) investigated for the first time the importance of optimization of surface coverage by DNA aptamer for efficient binding using Quartz Crystal Microbalance

with Dissipation mode (QCM-D). The aim of this study was to optimize the conditions of an EIS aptamer-based sensor for PSA detection. In fact, EIS optimisation for DNA aptamers is somewhat complex due to the different characteristics that induce a signal change: namely DNA density, change in charge density close to the electrode upon DNA conformational changes, size and charge of the analyte, screening of DNA charges upon analyte binding. The use of QCM-D provided valuable information about conditions for maximum analyte binding as well as the hydration, folding and behaviour of the aptamer distribution on the electrode. The system comprised a gold surface functionalized with a mixed SAM made of DNA aptamer and MCH which was used as spacer molecule. The best conditions in terms of buffer solution and aptamer mole fraction (concentration of aptamers/total thiols) for the binding of PSA to the aptamers were obtained by comparing the data from two techniques under similar conditions. With regards to the buffer conditions, the study demonstrated how the DNA aptamers' behaviour exhibits a strong dependence on the environment where it interacts with PSA.

In order to investigate an optimum surface chemistry that not only has a good antifouling effect but is also simple and cost effective, a new molecule has been investigated by Jolly et al. (2014) as a spacer molecule replacing MCH: a thiol terminated sulfo-betaine (fig 4). It was the first report on thiol terminated sulfo-betaine application for aptamer-based sensor. Thiol terminated sulfo-betaine, which has a molecular mass of 398.6 g/mole, is a zwitter ion due to presence of both positive and negative charges with a flexible chain that makes it a good antifouling molecule (see Fig 5). It been reported that sulfo-betaine not only reduces non-specific binding but also increases the sensitivity of the sensor (Bertok et al., 2013).

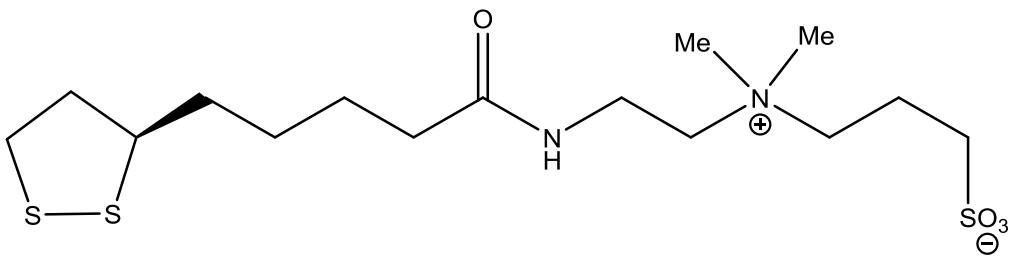

Fig. 4. Structure of thiol terminated sulfo-betaine. Image adapted from Bertok et al. (2013).

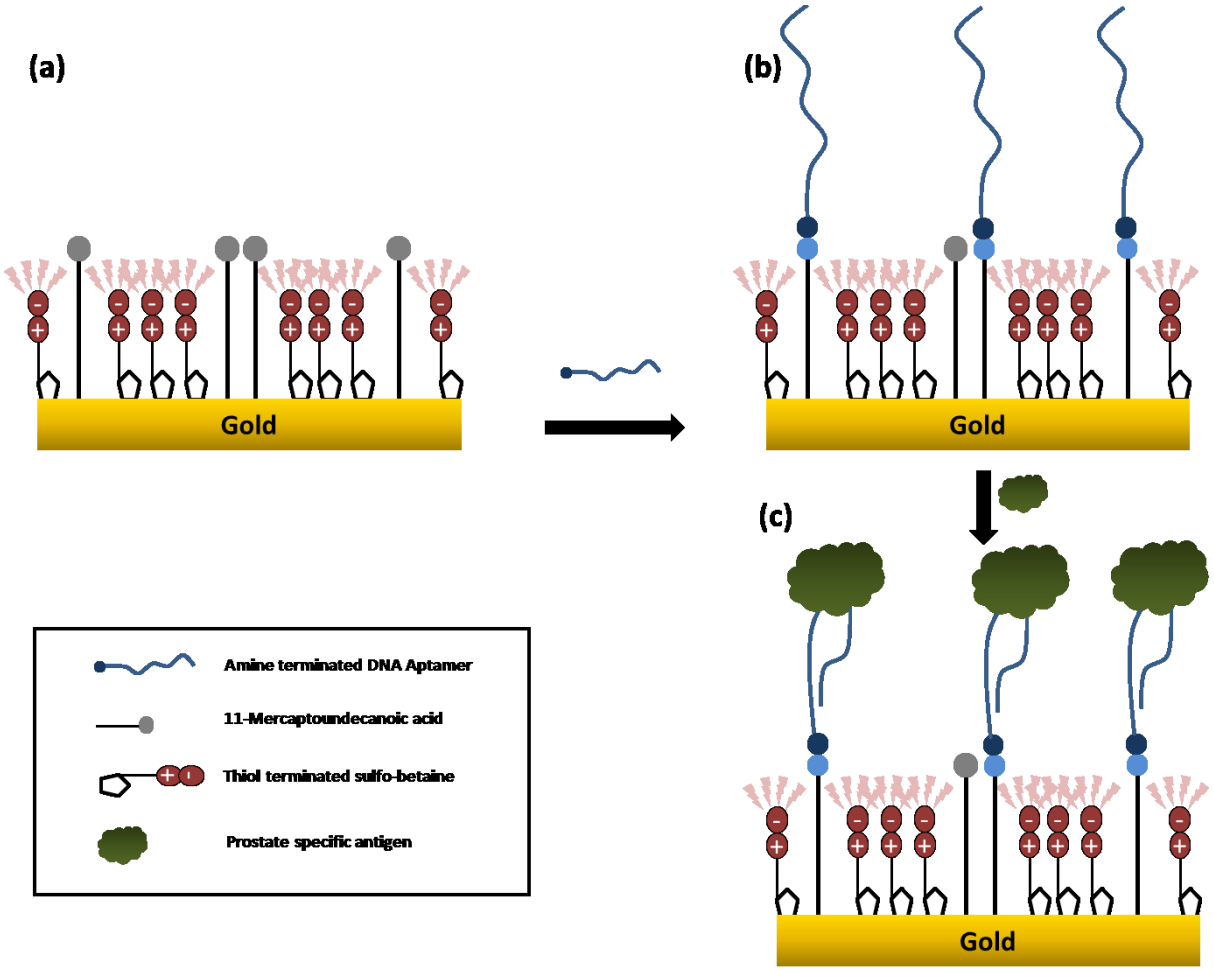

Fig. 5. Schematic of fabrication of thiol terminated sulfo-betaine based PSA aptasensor. (a) First SAM layer by co-immobilizing 11-mercatoundecanoic acid with thiol terminated sulfo-betaine. Image adapted from Jolly et al. (2014).

A comparison study between MCH and thiol terminated sulfo-betaine thiol chemistry was carried out by monitoring non-specific binding using human serum albumin (HSA) as a control protein. A schematic of the fabrication protocol for surface chemistry with thiol terminated sulfo-betaine is presented in fig 5. Co-immobilization of 11-mercaptoundecanoic acid (MUA) and thiol terminated sulfo-betaine formed the first SAM layer on clean gold electrodes. The carboxyl group of MUA was then activated with conventional EDC/NHS coupling reaction. The activated carboxyl groups were then used to immobilize amine terminated DNA aptamers for PSA and finally the electrodes were treated with ethanolamine to deactivate all the unreacted groups. The fabricated aptasensor with thiol terminated sulfo-betaine surface chemistry can discriminate PSA levels down to 1 ng/ml, which falls in the

lower clinical cut-off range of PSA in blood. The fabricated aptasensor with thiol terminated sulfo-betaine also showed a significant reduction of the non-specific binding with HSA as compared to the sensor where MCH was used instead as a spacer molecule. However, it has also been reported the obstacles on the optimization of the amount of DNA aptamers immobilized on the surface via EDC/NHS coupling. It was assumed that the charged thiol terminated sulfo-betaine has an influence on the attachment of DNA aptamer to activated MUA via EDC/NHS coupling leading to difference in amounts of DNA aptamers in different electrodes fabricated under similar conditions.

Aptasensors for other PCa biomarkers

Besides PSA, other biomarkers for PCa are currently studied and can potentially be used for DNA/RNA-based detection systems. One is the prostate-specific membrane antigen (PSMA), which is a type II integral membrane glycoprotein found in human serum. It is overexpressed on prostate tumour cells and may play an important role in the progression of PCa. It can also differentiate between BPH and PCa (Feneley et al., 2000; Ghosh and Heston, 2004; Madu and Lu, 2010; Pircher et al., 2011). Furthermore, by analysing the expression of PSMA, two cell lines can be distinguished among PCa cells: PSMA (-) and PSMA (+) cells (Ghosh and Heston, 2004). Min et al. (2010) reported on an RNA/peptide dual-aptamer-based biosensor able to detect both PSMA (-) and PSMA (+) cells by using EIS. The biosensor comprises of an anti-PSMA RNA aptamer (Lupold et al., 2002) which can target PSMA (+) cells and a DUP-1 peptide aptamer (Zitzmann et al., 2005) specific for PSMA (-) cells.

Another emerging biomarker is Alpha-methylacyl-CoA Racemase (AMACR), which is a racemace type of protein found in urine and blood. Its function is to metabolize fatty acids in the human body. It is also overexpressed in PCa and can be detected with a high sensitivity and specificity with a cut off value of 10.6 ng/ml. It also has the potential to differentiate between BPH and PCa. Currently AMACR aptamers have been independently developed by Base Pair Biotechnologies, Inc. (aptamer AM310_2) and by Yang et al. (2013). However, no reports on their application to biosensing have been published so far.

Future perspectives and conclusions

Recent work on the development of PSA aptasensors has enabled the transition from using antibody to aptamers as a recognition layer. Surface modification plays an important role in the development of promising biosensors which would be aided with the ongoing revolution in fabrication techniques. Easier fabrication would enable these biosensors to be mass produced and commercially viable. The inclination towards the development of aptasensors for PSA still needs further investigation for its use as an alternative to antibodies. Also, the sensitivity of an aptasensor is most likely to be influenced not only by the surface chemistry but also by the analytical method used for the detection of the target molecule, and so far no aptasensors have yet been used in complex samples such as blood. Overall, the development of aptamer based biosensor will see increasing reported literature because of its ease of synthesis and the possibilities of multiple modifications; it will always be a fresh field for more scope of adaptation of methodologies that will finally drive their solicitations with real blood samples. For early diagnosis of PCa, detection of different biomarkers would be preferred; consequently, more work is expected on development of aptamers for different isoforms of PSA and other biomarkers of PCa. An ideal biosensor for PCa detection would be based on a parallel sensing of different biomarkers using an array of sensors for more accurate diagnosis. In addition to the need for a simple surface chemistry, the scope of biosensor in future point-of-care devices will majorly depend on the integration of the format into a device that will enable easy and simple sample handling and an efficient read out system with rapid and accurate sample analysis of minimal blood sample volumes.

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